TRANSLATION NO. 2518

DATE: December 1966

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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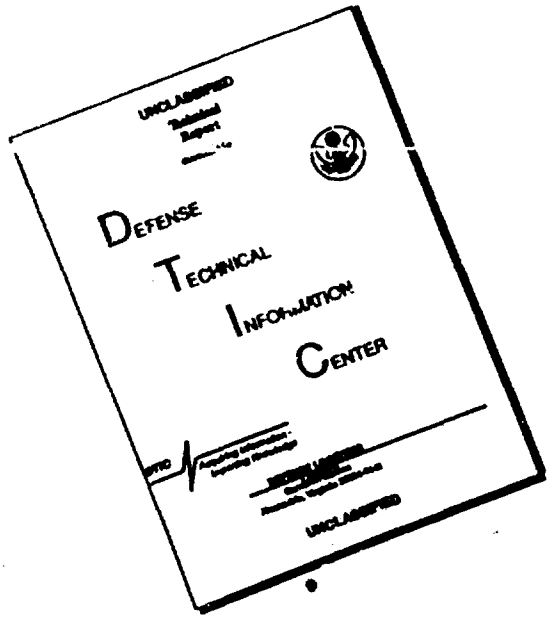

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PRODUCTION OF FLUORESCENT GAMMA-GLOBULINS USEFUL FOR CLINICAL AND LABORATORY DIFFERENTIAL DIAGNOSIS OF VIRAL DISEASES

[Following is the translation of an article by L. B. Mekler and V. K. Naumova, Institute of Virology imeni D. I. Ivanovskogo, AMN USSR, Moscow, published in the Russian-language periodical Voprosy Virusologii (Problems of Virology), Mar-Apr 1965, pages 235--240. It was submitted on 10 Jul 1964. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

The fluorescent antibody method has been put into practice more extensively as a method for the diagnosis of infectious diseases [5, 8, 13]. The feasibility of using this method, especially for the diagnosis of viral infections, has increased considerably as a result of the development of a method for the purification of fluorescent gamma-globulins with the help of chromatography on DEAE-cellulose* [7, 15, 16, 17]. The advantage of this method is the obtaining of conjugates, free from nonspecific fluorescence and stable during prolonged storage. Below we will describe 2 variations of the chromatographic method of purification, used by us for obtaining FITC-gamma-globulins** to the viruses of influenza, para-influenza and adenoviruses.

Materials and Methods

1./ Sera: Horse polyvalent hyperimmune sera to a/ viruses of influenza A, A1, A2, B, and B1; b/ viruses of para-influenza HA-1, HA-2, and CA; c/ adenoviruses of types 2, 3, 4 and 7. 2./ Viruses: Influenza type A, strain PR8, para-influenza HA-1, HA-2, and CA, adenoviruses of types 3 and 7. 3./ Tissue cultures: Transplantable line of monkey kidney cells [19]. 4./ The reagents and methods of analysis were described earlier [6]. 5./ Microscopy: The preparations for microscopy were prepared by the method [18] and studied on a ML-1 fluorescence microscope, using an objective of 90 x 1.25, eyepiece x4, and light filters 2S3S-7, 2-BS-8, 2-FS-1 and ZhO-18. Photography was carried out on RF-3 35-millimeter fluorographic film with a "Zenit-S" camera.

Results and Discussion

1. Obtaining of globulins and the conjugation reaction. The serum was diluted twice with distilled water, cooled to 0°, and a saturated solution of $(NH_4)_2SO_4$, pH 7.0, up to 40% saturation added. After 2 hours

* Diethylaminoethyl cellulose

** Fluoresceinisothiocyanate-gamma-globulin

solution of NaCl in 0.01 M phosphate buffer solution is fed into a blender mounted on a magnetic agitator. Under these conditions (the specified blender and concentration of NaCl) there is a noticeable linear and adequately sloping change in the concentration of NaCl in the solution feeding into the column (figures 2, 4). If there is an increase in the concentration of NaCl, FITC-globulins are dislodged from the column with ever increasing content of FITC in a molecule of FITC-globulin (figures 2, 3). A calculation of the content of FITC in a molecule of FITC-globulin, carried out by the method [6], showed that in such a manner it is possible to preparatively distinguisg molecules of conjugates in which 1 M of globulin contains respectively 1, 2 and 3 M of FITC. However, during chromatography on a column a significant weakening of the solution of FITC-globulin emerges. The concentration of protein is reduced to 0.01--0.02%, which often leads to the necessity to concentrate a purified solution of FITC-globulin. When working with antivirus sera, with which it is not always possible to obtain sufficiently high antibody titers, concentration is apparently a compulsory procedure. The latter may be avoided if column chromatography is replaced by adsorption with the damp powder of DEAE-cellulose.

Variant 2. In the analysis of the fractions obtained during gradient elution from a column of DEAE-cellulose it was shown that fractions, containing conjugates with a FITC: globulin ratio 2, are eluted at a NaCl concentration from 0.14 up to 0.25 M. Under these conditions conjugates which produce a nonspecific fluorescence, that is characterizing a FITC globulin ratio> 2, remain stably bound with DEAE-cellulose. Therefore it could be expected that the addition of DEAE-cellulose to a solution of conjugates, in which the concentration of NaCl was preliminarily brought to the required value, makes it possible to adsorb from the solution those conjugates containing more than 2 molecules of FITC per molecule of globulin. For determining the quantity of DEAE-cellulose, sufficient for the complete adsorption of the FITC-globulins subject to removal, NaCl up to a concentration of 0.14 M was added to a solution of "fraction I." Then to a volume of solution, cintaining FITC-globulins in a quantity corresponding to 100 mg of protein, we added 1 g of DEAE-cellulose, pressed out on a filter and preliminarily equilibrated with an 0.01 M phosphage buffer solution, pH 7.5. The solution of FITC-globulin was removed from the DEAE-cellulose by centrifuging, for which the mixture was placed in a Shott filter No 2 or No 3, set up in a stopper in the centrifuge vessel. During centrifuging the solution decants into the vessel and the DEAE-cellulose remains on the filter. A similar technique is preferable to filtration under a vacuum becuase it excludes foaming and the inactivation of antibodies connected with it. It can be seen that complete adsorption takes place with the addition of 4 g of DEAE-cellulose, pressed out on a filter, for each 100 mg of fraction I protein.

For proof that the described procedure removes FITC-globulins with a FITC:globulin molar ratio > 2, the resulting solution was subjected to chromatography on a column of DEAE-cellulose by the method of "variant 1"

(figure 4). It is apparent that after adsorption with DEAE-cellulose using variant 2, the solution contains only FITC-globulins with a FITC: globulin molar ratio of 1 and 2. The final preparation was poured into ampoules and stored in the dark at 4°.

Variant 3. Obtaining diagnostic FITC-globulins to the viruses of influenza, para-influenza and the adenoviruses.

For the purpose of a more thorough check of the FITC-globulins, obtained by the described method, small series of the above mentioned diagnostic preparations were prepared using variant 2. The typical results of analyzing the corresponding fractions, obtained during purification, are presented in tables 1 and 2.

It follows from tables 1 and 2 that the end preparation (see column 3) contains around 50% of the initial globulin. The loss of half of the original protein is connected with two circumstances. As was stated above, during gelfiltration through a Sephadex column dilution of the solution was observed. Desireing to increase the concentration of the solution, we did not use the first and the last (most diluted parts) fractions I, which lead to the loss of 20--30% of the original protein. This part of the solution may be concentrated and used. Other losses are connected with the kinetics of the conjugation reaction. Apparently the conditions selected in this work are close to optimum, becuase from each 4 molecules of globulin only one bound more than 2 molecules of FITC. From 10 to 20% of the initial globulin was contained in the fraction, eluting with an 0.2 M solution of NaCl. When working with variant 2, in contrast to variant 1, it contained a small quantity of FITC-globulins with a large content of FITC, which could lead to nonspecific fluorescence when staining preparations with this solution. Around 10% of the original globulin is contained in the fractions, eluted with 0.3 and 0.6 M solutions of NaCl. These fractions produced an expressed nonspecific staining [7, 16]. The final preparation contains 0.4--0.5% protein. Before staining preparations it should be diluted with a physiological solution, buffered with an 0.01 M phosphate buffer solution, pH 7.0. The degree of dilution is determined by the titer of antibodies. Thus the anti-influenza FITC-globulin (titer in the hemagglutination inhibition reaction 1:400) we diluted by 10, and the anti-para-influenza (titer 1:256) - by 5 times. Further, by staining tissue cultures infected with viruses, we determined the degree of specificity of staining with the corresponding preparations of FITC-globulins. It was assumed that a preparation produces only specific staining if the following conditions are fulfilled: 1) there is an absence of diffuse staining of cells not infected with the homologous virus; 2) there is an absence of diffuse staining of cells infected with heterologous virus; 3) during staining of cells infected with the homologous virus a clear picture of localization of the viral antigen is exposed; 4) the latter is not revealed if there is a preliminary blocking with untagged immune globulin. Typical photographs are shown in figures 5--9.

It is apparent that there is an absence of nonspecific luminescence during staining with a preparation adsorbed by DEAE-cellulose, and it is present during staining with a preparation which had passed only through a Spehadex G-50 column.

The FITC-globulins described were used for the differential diagnosis of respiratory viral infections of man. Two methods were tested: 1) direct exposure of viral antigens in the cells of the columnar epithelium of the nasopharynx mucosa of man [1--4], and 2) infection of the transplanted culture of monkey kidney cells with infectious material, obtained from a patient, with the subsequent exposure of the viral antigen 24 hours after infection of the culture [9, 10]. The study of materials, obtained during the investigation of more than 200 patients and model tests on tissue culture showed that the FITC-globulins obtained by the described method are suitable for carrying out the differential diagnosis of viral infections. The preparations of FITC-globulins preserved their immunological activity and specificity of staining for at least 5--6 months after they were obtained.

Conclusions

1. A chromatographic method of fractionation has been described for the isolation of FITC-globulins with the desired molar ratio of FITC: FITC-globulins.

2. It was verified that during staining of preparations with FITC-globulins with a FITC: globulin molar ratio 2 nonspecific fluorescence is absent.

3. It was demonstrated that with the help of the stated method it is possible to obtain FITC-globulins suitable for the differential diagnosis of viral infections and stable during prolonged storage.

Table 1

Fractionation of horse anti-influenza FITC-globulin.

Materials	Volume (in ml)	Concentration of protein (in %)	Concentration of FITC (in mg%)	Concentration of FITC globulin (in % from initial concentration)		Titer in the RZGA **	* $\frac{M_{FITC}}{M_{protein}}$
				Protein	FITC		
Original globulin	127	2.0	24.0	100	100	1:3200	7.1
Conjugate after purification on Sephadex G-50 column	300	0.67	3.75	79	37	1:800	2.85
Fraction, not adsorbed on DEAE-cellulose w/ 0.14 M solution of NaCl	300	0.39	1.92	47	19	1:400	2.23
Fractions, eluted with DEAE-cellulose w/:							
0.2 M NaCl solution	260	0.182	0.97	18.7	8.3	1:100	2.46
0.3 M NaCl solution	155	0.111	1.11	6.8	5.7	1:100	4.65
0.6 M NaCl solution	100	0.109	1.69	4.3	5.5	--	7.20

* Here and in table 2, M - molar concentration.
** RZGA - hemagglutination inhibition reaction.

Table 2

Fractionation of horse anti-adenovirus FITC-globulin.

Materials	Volume (in ml)	Concentration of protein (in %)	Concentration of FITC (in mg%)	Concentration of FITC-globulin (in % from initial concentration)		$\frac{M_{FITC}}{M_{protein}}$
				Protein	FITC	
Original globulin	30	2.09	24.0	100.0	100.0	7.1
Conjugate after purification on Sephadex G-50 column	67.5	0.65	4.0	70.5	37.5	2.85
Fraction, not adsorbed on DEAE-cellulose w/ 0.14 M solution of NaCl	67.5	0.485	2.03	52.0	19.0	1.78
Fractions, eluted with DEAE-cellulose w/:						
0.2 M NaCl solution	75.0	0.104	0.36	12.3	3.8	2.80
0.3 M NaCl solution	75.0	0.053	0.41	6.3	4.3	4.10
0.6 M NaCl solution	25.0	0.036	0.37	1.44	1.4	4.80

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Figure 1. Separation of FITC-globulins and FITC with the help of gel-filtration through a Sephadex G-50 column. Along the axis of ordinate - light passage at λ = 254 m , along the axis of abscissa - time or volume of solution passing through the column. 1 - FITC-globulin; 2 - FITC. Recorded with the help of a continuous photometer "Uvikord" from the LKB Firm, Sweden.

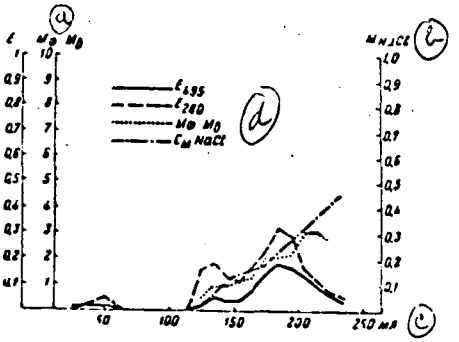

Figure 2. Fractionation of horse polyvalent anti-influenza FITC-globulin on a column of DEAE-cellulose. E - optical density; M_f/M_b - molar ratio of FITC:protein; C_m - molar concentration of NaCl.

a - $M_f \cdot M_b$; b - M_{NaCl}; c - ml; d - (from top to bottom): E_{495}; E_{280}; $M_f : M_b$; C_m NaCl.

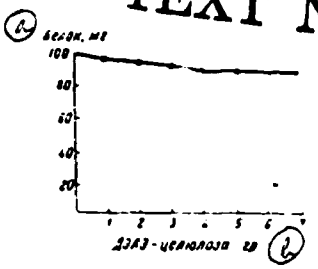

Figure 3. Adsorption by DEAE-cellulose of FITC-globulins with a FITC:protein molar ratio $>$ 2. Along the axis of ordinate - amount of protein in solution, along the axis of abscissa - amount of DEAE-cellulose, pressed out on a filter and preliminarily equilibrated with 0.01 M phosphate buffer solution, pH 7.5, added to the solution. Prior to adding DEAE-cellulose the amount of NaCl in the solution was 0.14 M. a - protein, mg; b - DEAE-cellulose, gr.

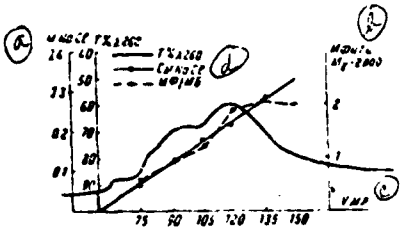

Figure 4. Control fractionation on a DEAE-cellulose column, using horse anti-influenza FITC-globulin with a FITC:: protein molar ratio $<$ 2 (end preparation). Conjugates with a molar ratio of FITC-:protein $>$ 2 are removed with the help of adsorption with DEAE-cellulose pressed out on a filter. On the curve for light passage are recorded, with the help of a "Uvikord" photometer, the results of the subsequent determination of the NaCl concentration and the molar ratio of FITC:protein (M_f/M_b). a - M NaCl T% λ 260; b - M_{FITC} and M_γ - glob/; c - V ml;d - (from top to bottom) T% λ260; C M NaCl ; M_f/M_b.

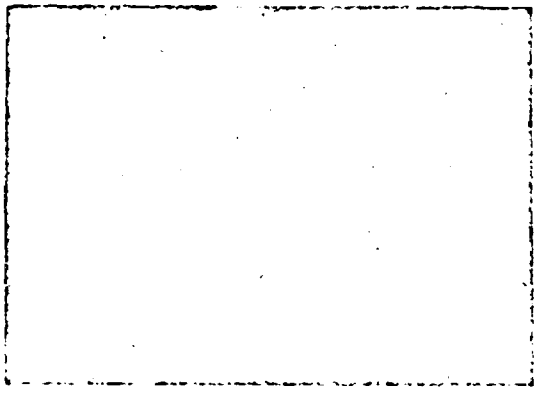

Figure 5. Transplanted culture of monkey kidney cells, infected with adenovirus type 3. 24 hours after infection. Stained with antiadenovirus FITC-globulin. Molar ratio FITC:protein. Protein concentration %.

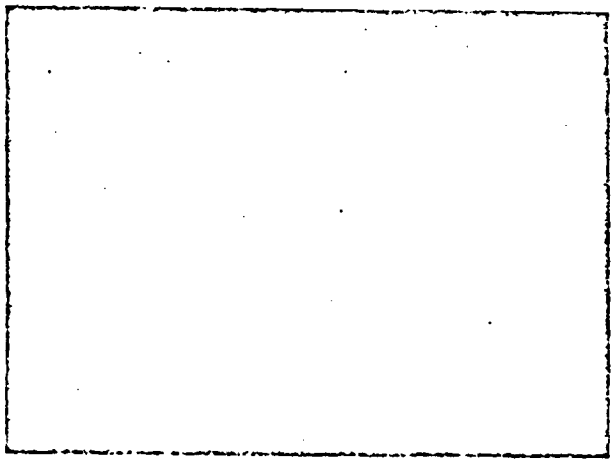

Figure 6. Similar preparation, but stained with FITC-globulin passed only through a column of Sephadex G-50.

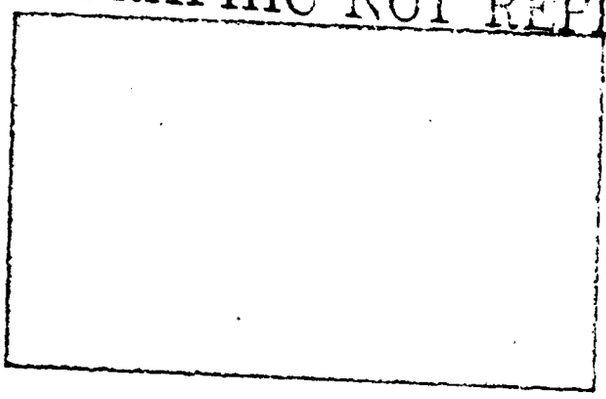

Figure 7. Similar preparation, but stained with anti-influenza FITC-globulin. Molar ratio FITC:protein. Protein concentration %.

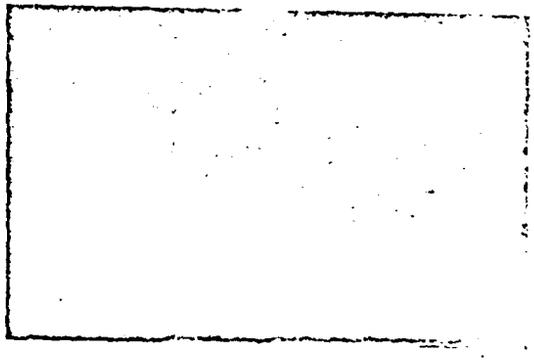

Figure 8. Noninfected preparation of culture, stained with antiadenovirus FITC-globulin. Molar ratio FITC:protein. Protein concentration %.

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Figure 9. Preparation of an analogous unstained culture.